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MONOCLONAL ANTIBODY TO AN ENDOGENOUS NEUROPEPTIDE WITH PUTATIVE MORPHINE-MODULATING ACTIVITY

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This technical report has been reviewed by the NMRI scientific and public affairs staff and is approved for publication. It is releasable to the National Technical Information Service where it will be available to the general public, including foreign nations.

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Monoclonal antibodies against phe-leu-phe-gln-pro-gln-arg-phe-NH_2 (F-8-F-NH_2), a FMRF-NH_2-like peptide of bovine brain, have been produced and characterized. The antibodies showed varying degrees of cross-reactivity with peptides having arg-phe-NH_2 at their C-termini but no cross-reactivity with met-enkephalin-arg-phe, NPY and PYY. The result suggests that these antbodies are directed to the C-terminal dipeptide, arg-phe-NH_2, and the conformation of the peptide also contributes to the immunoreactive potency. The result also indicates that these monoclonal antibodies are capable of differentiating-RF-NH_2, the C-terminal dipeptide amide of FMRF-NH_2-like peptides, from-RY-NH_2, the C-terminal dipeptide amide of NPY. An extract from bovine spinal cord was analyzed by HPLC coupled with RIA using the monoclonal antibodies. The major immunoreactivity was identified as F-8F-NH_2 although there were some very small amounts of unidentified immunoreactive species. The specificity of these monoclonal antibodies is thus further confirmed and will be useful in differentiating the distribution of F-8-F-NH_2 or mammalian FMRF-NH_2-like peptides from that of NPY by immunohistochemical studies. (KT/AC)

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The experiments reported herein were conducted according to the principles set forth in the guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, DHHS, publication No. (NIH) 86.23.

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INTRODUCTION

The cardioexcitatory peptide, phe-met-arg-phe-NH_2 (FMRF-NH_2), was originally isolated from ganglia of the mollusc, marcrocallista nimbosa (Price and Greenberg, 1977). In molluscs, this peptide was found to have a variety of biological properties including regulatory effects on the heart (Greenberg and Price, 1983), muscle (Painter et al., 1982) as well as modulatory effects on neurons (Colombaioni et al., 1985) and opioid peptides (Greenberg, et al., 1983).

FMRF-NH_2-like peptides have also been detected in CNS of various mammalian species using antisera raised against FMRF-NH_2 (Boer et al., 1980; Weber et al., 1981). Furthermore, in mice and rats, FMRF-NH_2 was found to exert many biological actions including the activity to modulate opiate effects (Kavaliers and Hirst, 1985; Kavaliers et al., 1985; Kavaliers and Hirst, 1986; Tang et al., 1984), excite brain neurons (Gayton, 1982), elevate blood pressure (Mues et al., 1982), induce depolarizing and hypopolarzing responses in cultured mouse spinal neurons, (Guzman et al., 1987) and induce excessive grooming behavior (Raffa et al., 1986). However, the mammalian FMRF-NH_2 immunoreactivity is now known to consist of multiple molecular forms which are not identical to FMRF-NH_2 (Chronwall et al., 1984; Dockray, 1985). Previously, we have isolated and chemically characterized a FMRF-NH_2-like peptide of bovine brain (Yang, et al., 1985). This peptide, phe-leu-phe-gln-pro-gln-arg-phe-NH_2 (F-8-F-NH_2), can attenuate the morphine induced prolongation of tail flick latencies in

the rat when injected intraventricularly (Yang, et al., 1985) and elevate arterial pressure when injected intravenously (Roth et al., 1987). In bovine CNS, F-8-F-NH_2 was found to be highly localized in dorsal spinal cord by radioimmunoassay (Majane and Yang, 1987). In order to further explore the functional role of F-8-F-NH_2, monoclonal antibodies to F-8-F-NH_2 were developed and characterized by radioimmunoassay techniques. The results suggest that these monoclonal antibodies are capable of differentiating F-8-F-NH_2 or F-8-F-NH_2-like peptides from neuropeptide Y (NPY) which is structurally similar to F-8-F-NH_2 or FMRF-NH_2 in its C-terminal sequence. In fact, it is difficult to differentiate FMRF-NH_2-like peptides from NPY immunohistochemically with some polyclonal antisera (Sasek and Elde, 1985).

MATERIALS AND METHODS

Materials:

F-8-F-NH_2, pro-gln-arg-phe-NH_2 (P-4-F-NH_2) and ala-gly-glu-gly-leu-ser-ser-pro-phe-trp-ser-leu-ala-ala-pro-gln-arg-phe-NH_2 (A-18-F-NH_2) were custom synthesized by Peninsula Laboratory, Inc. (Belmont, CA), and purified by HPLC. FMRF-NH_2, met^5-enk-arg^6-phe^7, arg-phe-NH_2 and γ_1-melanocyte stimulating hormone (γ_1-MSH) were purchased from Peninsula Laboratory, Inc. Neuropeptide Y (NPY) and peptide YY (PYY) were purchased from Bachem (Torrance, CA).

Immunization of mice:

F-8-F-NH_2, 6 mg, and keyhole limpet hemocyanin, 5 mg, were dissolved in 1 ml H_2O. To this solution, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 6 mg in 0.1 ml H_2O, was added. After 16 hrs standing at room temperature, the reaction mixture was dialyzed against H_2O overnight and lyophilized. The conjugate was dissolved in saline and then emulsified in complete Freund's adjuvant (Cal. Biochem). Balb/c mice were injected subcutaneously with the F-8-F-NH_2-hemocyanin conjugate, 100 μg in 0.1 ml emulsion, once a week for four weeks. Three days prior to sacrifice,the mouse received the last immunization with the same dose of the conjugate.

Cell fusion and cloning:

Spleen cells (5×10^7) from immunized mice were fused with SP2/0 mouse myeloma cells (5×10^7) at log phase by polyethylene glycol 1500 (Boehringer Mannheim Biochemicals) as previously described (Galfrè and Milstein, 1981). Fused cells were resuspended in 60 ml of RPMI 1640 (Quality Biological, Inc.) culture medium containing hypoxanthine 13.6 mg/l, aminopterine 0.19 mg/l and thymidine 3.875 mg/l (HAT selective medium) and then distributed into 96-well microtiter plates (50 μl cell suspension and 200 μl HAT selective medium/wells). After incubation at 37°C in a CO_2 incubator for 2 days, 200 μl of HAT selective medium in each well was replaced with 250 μl of RPMI 1640 culture medium containing hypoxanthine, 13.6 mg/l, and thymidine. 3.875 mg/l, (HT hybridoma growth medium). Ten days later, tissue culture media from wells with hybridoma

colonies were screened for antibody to F-8-F-NH_2 by enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA). Cells from positive wells were cloned by limiting dilution (Galfrè and Milstein, 1981). Selected cloned hybridomas were preserved in a medium containing 70% RPMI culture medium, 20 % fetal calf serum and 10% DMSO and subsequently used for production of antibodies by tissue culture or by generation of ascitic fluid in mice.

ELISA method:

F-8-F-NH_2, 100 μg, was conjugated to 10 mg of bovine serum albumin (BSA) by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The F-8-F-NH_2-BSA conjugate was dialyzed against H_2O overnight and a total of 2 ml was obtained. The F-8-F-NH_2-BSA conjugate was diluted 1:10,000, and then dispensed into each well of microtiter plates (50 μl/well). After incubating at 4° C for 1 hour, wells were emptied, treated with 100 μl 0.5% BSA for 1 hour at 4°C and washed with 3 x100 μl phosphate buffered saline containing 0.04% Triton (PBS-Triton). Spent culture medium that is to be tested was dispensed into duplicate wells (50 μl/well); plates were incubated at 4°C for 1 hr and washed 3 times with 100 μl of PBS-Triton. Goat-anti mouse IgG conjugated to alkaline phosphatase (Sigma) was diluted with PBS-Triton (1:1,000), 50μl of this solution was added to each well and plates were incubated for another hour at 4°C. After repeated (7 times) washings with 100 μl PBS-Triton, 50 μl of p-nitrophenyl phosphate (0.5 mg/ml in 0.05 M Na_2CO_3 and 0.001 M $MgCl_2$, pH 9.8) was added and

plates were incubated at 37°C for 1.5 hr. The reaction was terminated by the addition of 50 μl 2 N NaOH into each well and results were read by an ELISA reader (Dynatech).

Radioimmunoassay:

Peptide, tyr-leu-phe-gln-pro-gln-arg-phe-NH_2 (Y-8-F-NH_2) was custom synthesized by Peninsula Laboratory and purified by reverse phase HPLC. Y-8-F-NH_2 was radiolabelled with Na[^{125}I] by chloramine T method (McConahey and Dixon, 1980) and purified by BioGel P-2 column chromatography developed with 1 N CH_3COOH containing 0.1% BSA.

For RIA, F-8-F-NH_2 standards or samples were incubated with [^{125}I]-Y-8-F-NH_2 (12,000-15,000 CPM) and antibody in 0.5 ml 0.2 M Tris buffer pH 7.4 containing 0.1% BSA, 0.06% dextran (RIA grade, Schwartz/Mann, Spring Valley, NY) in polystyrene tubes overnight at 4°C. Free [^{125}I]-Y-8-F-NH_2 was separated from [^{125}I]-Y-8-F-NH_2 bound to the antibody by adding 200 μl of RIA buffer containing 1.5% charcoal. The samples were centrifuged and 0.55 ml supernatants removed and counted in a Beckman biogamma counter.

Isotyping:

The isotyping of the monoclonal antibodies was performed by the sandwich ELISA method with the isotyping kit from Calbiochem (Cat No 386445) following the procedures described by the manufacturer.

Ascitic fluids:

Those hybridomas tested positive in the ELISA were further screened with RIA using [^{125}I]-Y-8-F-NH_2. The cells of the positive clones were injected (10^6 cells/injection) into the peritoneal cavities of female BALB/c mice primed with 0.5 ml pristane 5 days earlier, and 10-15 days later ascitic fluid was collected.

HPLC fractionation of spinal cord extract:

Bovine cervical spinal cord obtained fresh from a local slaughter house was transported on ice to the laboratory and the dorsal grey region was dissected out. Sprague Dawley rats (200 gms) were decapitated and spinal cords removed. Tissues were homogenized in 10 volume of 1 N CH_3COOH containing 0.02 N HCl by an Ultra-Turax tissumizer (Tekmar, Cincinnati, OH). The homogenates were heated for 10 min in a 100°C water bath and centrifuged at 15,000 x g for 20 min. The pellet was reextracted with 5 volume solution of 1 N acetic acid containing 0.02 HCl. The supernatants from the first and second extractions were combined and passed through a reverse phase C-18 cartridge (Waters Associate, Milford, MA). The C-18 cartridge was washed with 20 ml H_2O and then eluted with 6 ml of 60% CH_3CN containing 0.1% trifluoroacetic acid (TFA). The eluate was lyophilized and redissolved in H_2O for HPLC analysis using an Altex ODS 5 column (4.6 x 250 mm). The reverse phase column was eluted at a flow rate of 1 ml/min with a linear gradient of CH_3CN (20-50%/60 min) formed by CH_3CN containing 0.1% TFA and 0.1% TFA in H_2O. Fractions were collected every min and aliquots

from 1 ml fractions were dried in a vacuum concentrator (Savant, Speed Vac) and radioimmunoassayed with monoclonal antibody containing ascitic fluid. The ascitic fluid was used at a final dilution of 1:275,000.

RESULTS

Production of antibodies:

Three clones of hybridomas secreting monoclonal antibodies against F-8-F-NH_2 were identified after screening with ELISA and RIA and designated X10M1, X13M1 and X30S1. The isotypes of these antibodies were determined to be IgG_1 for X10M1 and X13M1 and IgG_{2b} for X30S1. The ascitic fluids were produced by injecting the hybridomas into the peritoneal chamber of pristane-primed mice. Titers of these three antibodies (ascitic fluids) determined by RIA using [^{125}I]-Y-8-F-NH_2 are shown in Fig. 1. Similar titers were observed for all three ascitic fluids. The antibodies (ascitic fluid) at final dilutions of 1:200,000-312,000 were used in RIA.

Antibody specificity:

The specificity of the antibody was examined by inhibition of [^{125}I]-Y-8-F-NH_2 binding to monoclonal antibodies by various peptides. The characteristics of the monoclonal antibody, X13M1, is shown in Fig. 2. All of the peptides having arg-phe-NH_2 at their C-termini cross-reacted with the antibody and their rank order of immunoreactive potencies are F-8-F-NH_2 > A-18-F-NH_2 > P-4-F-NH_2 > FMRF-NH_2 > γ_1-MSH > arg-phe-NH_2. The antibody showed no cross-reaction with neuropeptide Y (NPY), peptide YY (PYY) and met^5-enkephalin-arg^6-phe^7. The relative immunoreactivities of these peptides expressed in $(B/Bo)_{50}$ (concentrations of peptides required to displace 50% of the [^{125}I]-Y-8-F-NH_2 bound to the antibody) is

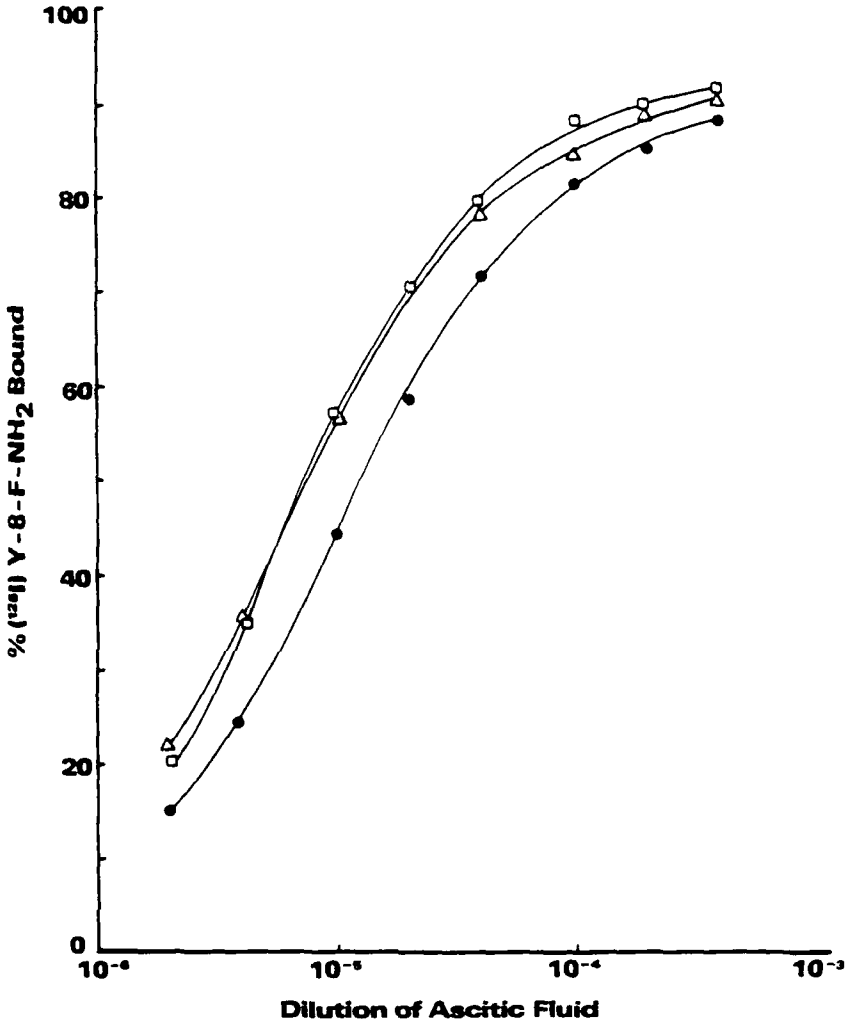
100
80
60
40
20
0
% (^{125}I) Y-8-F-NH$_2$ Bound
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Dilution of Ascitic Fluid

FIGURE 1

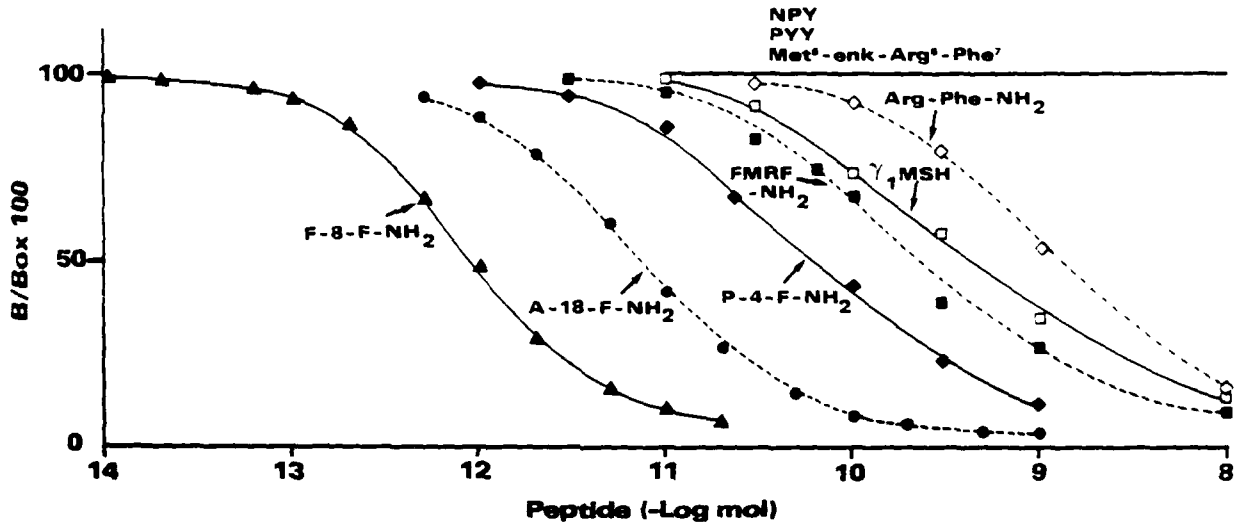
NPY
PYY
Met⁵-enk-Arg⁶-Phe⁷
Arg-Phe-NH2
FMRF-NH2
γ1MSH
F-8-F-NH2
A-18-F-NH2
P-4-F-NH2
B/Box 100
100
50
0
14
13
12
11
10
9
8
Peptide (-Log mol)

FIGURE 2

summarized in Table 1. Very similar specificities were observed with antibodies X10M1 and X30S1 (data not shown). The limit of RIA sensitivity is 50-100 fmol/sample (Fig. 2) under the RIA conditions used.

RIA of tissue extracts fractionated by HPLC:

To characterize further the monoclonal antibody, extracts from bovine and rat spinal cords were fractionated by reverse phase HPLC and radioimmunoassayed with the monoclonal antibody X13M1. In the bovine spinal cord extract, one major immunoreactive peak eluting in the position of synthetic F-8-F-NH_2 was obtained, although there were some minor immunoreactive peaks (Fig. 3 top). One of the minor immunoreactivities was eluted in the position of synthetic A-18-F-NH_2 and is most likely due to the cross-reactivity of this peptide with the antibody. In the rat spinal cord extract, one major peak and other minor immunoreactivities were obtained. The major peak was eluted with a retention time higher than that of synthetic F-8-F-NH_2 (Fig. 3, bottom).

TABLE 1

Cross-reactivity of F-8-F-NH_2 antibody with structurally related and unrelated peptides

Peptides	Number of Amino acid	Amino acid sequence	(B/B_o) 50 pmol/tube
F-8-F-NH_2	8	Phe–Leu–Phe–Gln–Pro–Gln–Arg–Phe–NH_2	0.9
A-18-F-NH_2	18	...Ser–Leu–Ala–Ala–Pro–Gln–Arg–Phe–NH_2	7
PQRF-NH_2	4	Pro–Gln–Arg–Phe–NH_2	60
FMRF-NH_2	4	Phe–Met–Arg–Phe–NH_2	200
γ_1-MSH	11	...Gly–His–Phe–Arg–Trp–Asp–Arg–Phe–NH_2	500
Arg-Phe-NH_2	2	Arg–Phe–NH_2	1000
Met^5-Enk-Arg^6-Phe^7	7	Tyr–Gly–Gly–Phe–Met–Arg–Phe–OH	$> 10^{4*}$
Neuropeptide Y	36	...Asn–Leu–Ile–Thr–Arg–Gln–Arg–Tyr–NH_2	$> 10^{4*}$
Peptide YY	36	...Asn–Leu–Val–Thr–Arg–Gln–Arg–Tyr–NH_2	$> 10^{4*}$

$(B/B_o)_{50}$: Amount of peptide required to displace 50% of [I^{125}]-Y-8-F-NH_2 bound to the antibody.

*: *Peptides which showed no reactivities with the antibody at 10,000 pmol/tube.*

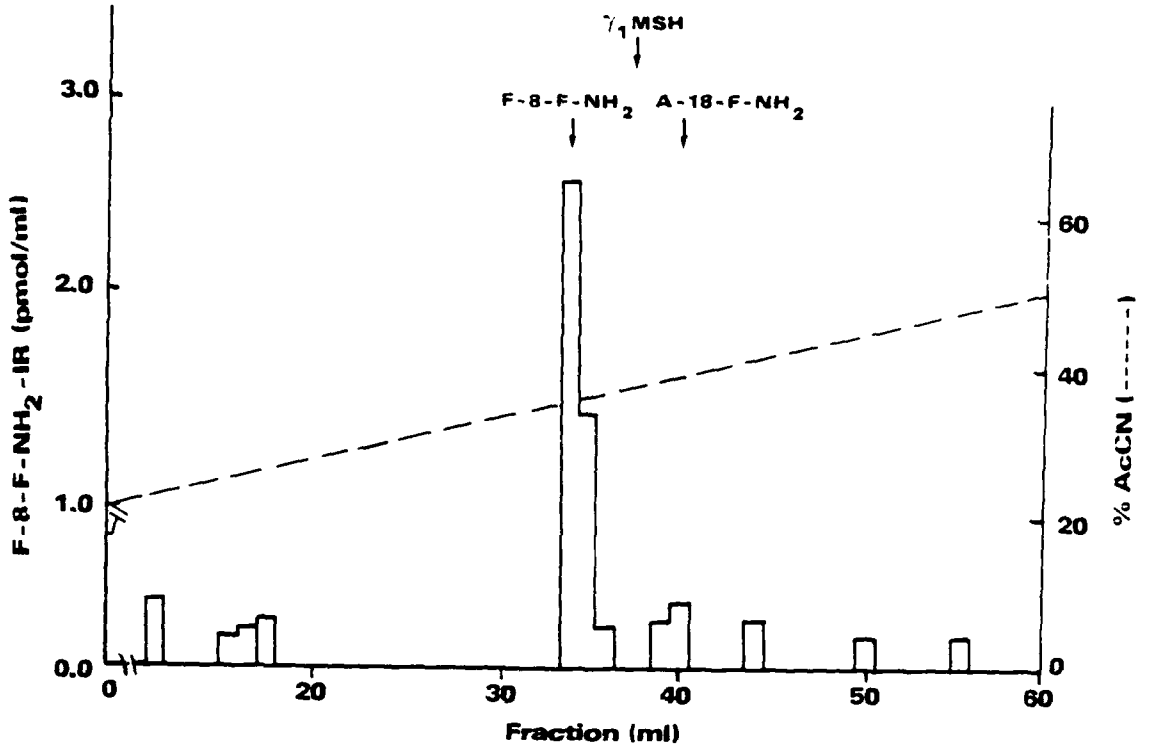

FIGURE 3 (top)

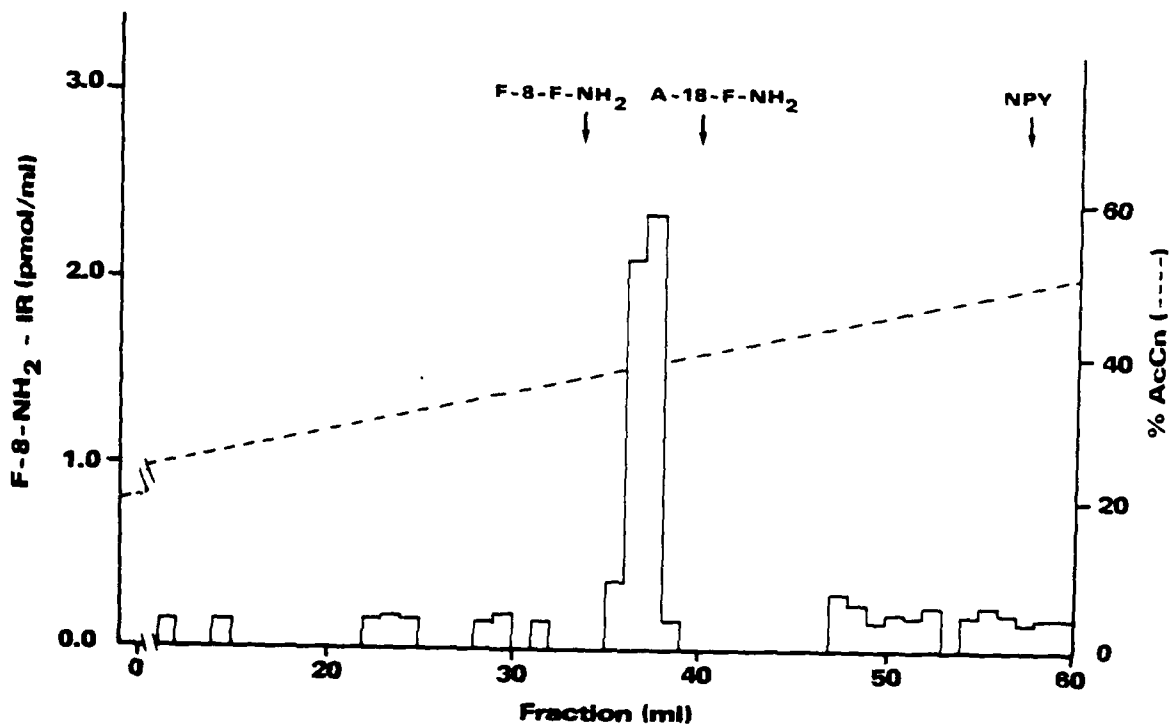

FIGURE 3 (bottom)

DISCUSSION

Three clones of hybridomas secreting antibodies to F-8-F-NH_2 were produced using F-8-F-NH_2 conjugated to hemocyanin as immunogen. From these hybridomas, high titer ascitic fluids were obtained (Fig. 1). The antibodies (ascitic fluid) showed varying degrees of cross-reactivities with peptides which share the same C-terminal dipeptide, arg-phe-NH_2 with F-8-F-NH_2. In contrast, the antibodies were found to be inactive to structurally non-related peptides including NPY. The results suggest that the antibodies are directed to the C-terminal dipeptide arg-phe-NH_2 but the conformation of the peptide appears to also contribute to the immunoreactive potency of the peptide.

The limit of the RIA sensitivity using the monoclonal antibody X13M1 is 50-100 fmol/sample which is 10-20 times lower than the sensitivity obtainable in the RIA with polyclonal antiserum raised in rabbits (Majane and Yang, 1987). In spite of this low RIA sensitivity, ascitic fluids were found to have very high titers (Fig. 1).

Polyclonal antiserum to FMRF-NH_2 or F-8-F-NH_2 has been found to cross-react with NPY weakly (Sasek and Elde, 1985; Majane and Yang, 1987). Because of this and the abundance of NPY in mammalian CNS, it may be difficult, sometimes, to differentiate FMRF-NH_2-like peptides including F-8-F-NH_2 from NPY by immunohistochemical studies with polyclonal antisera (Sasek and Elde, 1985). The monoclonal antibody to F-8-F-NH_2 is unreactive to NPY (fig. 2) and this specificity is further confirmed by the analysis

of spinal cord F-8-F-NH_2 immunoreactivity after the HPLC fractionation. As shown in Fig. 3, the monoclonal antibody seems to detect one major immunoreactivity in the extract from spinal cord of bovine or rat although some very minor immunoreactivities are revealed. The F-8-F-NH_2 immunoreactivity in the rat spinal cord appears to be more hydrophobic than synthetic F-8-F-NH_2 suggesting an interspecies molecular heterogeneity.

The characteristics of the monoclonal antibody to F-8-F-NH_2 produced in this study strongly suggest that this antibody will be very useful in studying the distribution of F-8-F-NH_2 exclusive of NPY in the mammalian CNS. Several lines of evidence suggest that F-8-F-NH_2 may have a modulatory role in opiate mediated analgesia (Tang et al., 1984; Yang et al., 1984 and 1985; Majane and Yang 1987), however, the precise physiological role of F-8-F-NH_2 or FMRF-NH_2-like peptides in mammalian CNS still remain unclear. Immunohistochemical studies on the distribution of F-8-F-NH_2 without interference from NPY, which is highly abundant in the mammalian CNS, may lay a frame work for the further exploration of the role of F-8-F-NH_2 or FMRF-NH_2-like peptide in neuronal function.

REFERENCES

Boer H.H., Schot L.P.C., Veenstra J.A., and Reichelt D. (1980) Immunocytochemical identification of neural elements in the central nervous system of a snail, some insects, and a mammal, with an antiserum to the molluscan cardioexcitatory tetrapeptide FMRF-amide. Cell Tissue Res. **213**, 21-27.

Chronwall B.M., Olschowka J.A., and O'Donohue T.L. (1984) Histochemical localization of FMRFamide-like immunoreactivity in the rat brain. Peptides **5**, 563-584.

Colombaioni L., Paupardin-Tritsch D., Vidal P.P., and Gerschenfeld H.M. (1985) The neuropeptide FMRF-amide decreases both the Ca^{++} conductance and a cyclic 3'-5'-adenosine-monophosphate-dependent K^{+} conductance in identified molluscan neurons. J. Neurosci. **9**, 2533-2538.

Cottrell G.A., Davies N.W., and Green K.A. (1984) Multiple actions of a molluscan cardioexcitatory neuropeptide and related peptides in identified Helix neurones. J. Physiol. **356**, 315-333.

Galfrie G. and Milstein C. (1981) Preparation of monoclonal antibodies: Strategies and procedures, in Methods in Enzymology, Vol. 73: Immunochemical Techniques. (Langone J.J. and Vunakis H.V., eds), pp. 3-47. Academic Press, New York.

Gayton R.J. (1982) Mammalian neuronal actions of FMRFamide and the structurally related opioid met-enkphalin-arg^{6}-phe^{7}. Nature **298**, 275-276.

Greenberg M.J., Painter S.D., Dole K.E., Nagle G.T., Price D.A and Lehman H.K. (1983) The molluscan neurosecretory peptide FMRFamide: Comparative pharmacology and relationship to the enkephalins. Fed. Proc. **42**, 82-86.

Greenberg M.J. and Price D.A. (1983) Invertebrate neuropeptides: native and naturalized. Ann. Rev. Physiol. **45**, 271-288.

Guzman A., Legendre P., Demostes F., Geoffre S., Precignioux G., Vincent J.D., and Simonnet G. (1987) Effect of morphine modulating peptide (FLFQPQRF-NH_2) on electrophysiological properties of cultured mouse spinal cord cells. Soc. Neurosci Abst 13: 769.

Kavaliers M. and Hirst M. (1985) FMRFamide suppresses kappa opiate induced feeding in the mouse. Peptides **6**, 847-849.

Kavaliers M. and Hirst M. (1985) FMRFamide, a putative endogenous opiate antagonist: evidence from suppression of defeat-induced analgesia and feeding in mice. Neuropeptides **6**, 485-494.

Kavaliers M., Hirst M., and Mathers A. (1985) Inhibitory influences of FMRFamide on morphine- and deprivation-induced feeding. Neuroendocrin. **40**, 533-535.

Kavaliers M. and Hirst M. (1986) Inhibitory influences of FMRFamide and PLG on stress-induced opioid analgesia and activity. Brain Res. **372**, 370-374.

Majane E.A. and Yang H.-Y.T. (1987) Distribution and characterization of two putative endogenous opioid antagonist peptides in bovine brain. Peptides **8**, 657-662.

McConahey P.J. and Dixon F.J. (1980) Radioiodination of proteins by the use of the chloramine-T method in Methods in Enzymology, Vol. 70: Immunochemical Techniques. (Vunakis H.V. and Langone J.J., eds.) pp. 210-211. Academic Press, New York.

Mues G., Fuchs I., Wei E.T., Weber E., Evans C.J., Barchas J.D., and Chang J.-K. (1982) Blood pressure elevation in rats by peripheral administration of tyr-gly-gly-phe-met-arg-phe and the invertebrate neuropeptide, phe-met-arg-phe-NH_2. Life Sci. **31**, 2555-2561.

Painter S.D., Morley J.S. and Price D.A. (1982) Structure-activity relations of the molluscan neuropeptide FMRFamide on some molluscan muscles. Life Sci. **21**, 2471-2478.

Price D.A. and Greenberg M.J. (1977) The structure of a molluscan tatory neuropeptide. Science **197**, 670-671.

Roth B.L., Disimone J., Majane E.A., and Yang H.-Y.T. (1987) Elevation of arterial pressure in rats by two new vertebrate peptides FLFQPQRF-NH_2 and AGEGLSSPFWSLAAPQRF-NH_2 which are immunoreactive to FMRF-NH_2 antiserum. Neuropeptides **10**, 37-42.

Sasek C.A. and Elde R.P. (1985) Distribution of neuropeptide Y-like immunoreactivity and its relationship to FMRF-amide-like immunoreactivity in the sixth lumbar and first sacral spinal cord segments of the rat. J. Neurosci. **5**, 1729-1739.

Tang J., Yang H.-Y.T., and Costa E. (1980) Inhibition of spontaneous and opiate-modified nociception by an endogenous neuropeptide with phe-met-arg-phe-NH_2-like immunoreactivity. Proc. Natl. Acad. Sci. USA **81**,

5002-5005.

Telegdy G. and Bollók I. (1987) Amnesic action of FMRFamide in rats. Neuropeptides **10**, 157-163.

Weber E.C., Evans C.J., Samuelsson S.J., and Barchas J.D. (1981) Novel peptide neuronal system in rat brain and pituitary. Science **214**, 1248-1250.

Yang H.-Y.T., Fratta W., Majane E.A., and Costa E. (1985) Isolation, sequencing, synthesis and pharmacological characterization of two brain neuropeptides that modulate the action of morphine. Proc. Natl. Acad. Sci. USA **82**, 7757-7761.

Yang H.-Y.T., Tang J. Iadarola M. Panula P., and Costa E. (1985) Are phe-met-arg-phe-NH_2 immunoreactive peptides endacoids modulating opiate antinociception? in Endacoids (Lal H., ed.) pp. 313-322. Raven Press, New York.

FIG. 1 Titer of the ascitic fluids.

[^{125}I]-Y-8-F-NH$_2$ (20,000 CPM) was incubated with various dilutions of ascitic fluids X10M1 (●—●), X13M1 (□—□) or X30S1 (△—△) in 0.5 ml 0.2 M Tris buffer pH 7.4 containing 0.1% BSA, 0.06% dextran in polystyrene tubes overnight at 4°C. Free [^{125}I]-Y-8-F-NH$_2$ was separated from [^{125}I]-Y-8-F-NH$_2$ bound to antibody by adding 200 µl of RIA buffer containing 1.5% charcoal followed with centrfugation. The result is expressed as % [^{125}I]-Y-8-F-NH$_2$ bound to the antibody.

FIG. 2 RIA specificity.

Competitive displacement of [^{125}I]-Y-8-F-NH$_2$ bound to monoclonal antibody X13M1 by F-8-F-NH$_2$ (▲—▲), A-18-F-NH$_2$ (●—●), P-4-F-NH$_2$ (◆—◆), FMRF-NH$_2$ (■—■), γ_1-MSH (□—□), arg-phe-NH$_2$ (◇—◇), NPY, PYY and met^5-enk-arg^6-phe^7. The results are expressed as percentage of sample reacted in the absence of competition. B/B$_o$ is the ratio of the specific binding in the presence of non-labelled peptide to the maximal specific binding.

FIG. 3 High pressure liquid chromatography of F-8-F-NH$_2$ immunoreactivity in cervical dorsal grey region of bovine spinal cord (top) and rat spinal cord (bottom).

Extracts from 1.5 g bovine cervical dorsal grey or 0.6 g rat spinal cord were concentrated using a C-18 cartridge as described in the text and

applied to an Altex ultrasphere ODS-5 column (4.6x250 mm) eluted with a linear gradient of 20-50% CH_3CN in 0.1% trifluoroacetic acid over 60 min at a flow rate of 1 ml per min. Aliquots from 1 ml fractions were radioimmunoassayed with F-8-F-NH_2 monclonal antibody X13M1.

ABBREVIATIONS:

CNS,	central nervous system
FMRF-NH_2,	phe-met-arg-phe-NH_2
F-8-F-NH_2,	phe-leu-phe-gln-pro-gln-arg-phe-NH_2
NPY,	neuropeptide Y
A-18-F-NH_2,	ala-gly-glu-gly-leu-ser-ser-pro-phe-trp-ser-leu-ala-ala-pro-gln-arg-phe-NH_2
P-4-F-NH_2,	pro-gln-arg-phe-NH_2
HPLC,	high pressure liquid chromatography
γ_1-MSH,	γ_1-melanocyte stimulating hormone
PYY,	neuropeptide YY
HAT,	hypoxanthine, aminopterine, thymidine
HT,	hypoxanthine, thymidine
ELISA,	enzyme-linked immunosorbent assay
RIA,	radioimmunoassay
DMSO,	dimethylsulfoxide
BSA,	bovine serum albumin
PBS,	phosphate buffered saline
Ig,	immunoglobulin
TFA,	trifluoroacetic acid